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(54) Title: PEPTIDE-BASED GEMINI COMPOUNDS

(57) Abstract

New peptide-based gemini compounds comprising two linked chains (a) each chain having: (1) a positively charged hydrophilic head, $Q^1$ or $Q^2$, formed from one or more amino acids and/or amines, (2) a central portion, $P^1$ or $P^2$, having a polypeptide backbone, and (3) a hydrophobic tail, $R^1$ or $R^2$, the central sections of each chain being linked together by bridge Y through residues in $P^1$ and $P^2$, are disclosed. Methods for their preparation and uses are also disclosed. Such uses include transfection of polynucleotides into cells *in vivo* and *in vitro*.

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# PEPTIDE-BASED GEMINI COMPOUNDS

This invention relates to newly identified peptide-based gemini surfactant compounds, to the use of such compounds and to their production. The invention also relates to the use of the peptide-based gemini compounds to facilitate the transfer of compounds into cells for drug delivery.

Surfactants are substances that markedly affect the surface properties of a liquid, even at low concentrations. For example surfactants will significantly reduce surface tension when dissolved in water or aqueous solutions and will reduce interfacial tension between two liquids or a liquid and a solid. This property of surfactant molecules has been widely exploited in industry, particularly in the detergent and oil industries. In the 1970s a new class of surfactant molecule was reported, characterised by two hydrophobic chains with polar heads which are linked by a hydrophobic bridge (Deinega,Y *et al., Kolloidn. Zh.* 36, 649, 1974). These molecules, which have been termed "gemini" (Menger, FM and Littau,CA, *J.Am.Chem.Soc.* **113**, 1451, 1991), have very desirable properties over their monomeric equivalents. For example they are highly effective in reducing interfacial tension between oil and water based liquids and have a very low critical micelle concentration.

Cationic surfactants have been used *inter alia* for the transfection of polynucleotides into cells in culture, and there are examples of such agents available commercially to scientists involved in genetic technologies (for example the reagent Tfx™-50 for the transfection of eukaryotic cells available from Promega Corp. WI, USA).

The efficient delivery of DNA to cells *in vivo*, either for gene therapy or for antisense therapy, has been a major goal for some years. Much attention has concentrated on the use of viruses as delivery vehicles, for example adenoviruses for epithelial cells in the respiratory tract with a view to corrective gene therapy for cystic fibrosis (CF). However, despite some evidence of successful gene transfer in CF patients, the adenovirus route remains problematic due to inflammatory side-effects and limited transient expression of the transferred gene. Several alternative methods for *in vivo* gene delivery have been investigated, including studies using cationic surfactants. Gao,X *et al.* (1995) *Gene Ther.* 2, 710-722 demonstrated the feasibility of this approach with a normal human

gene for CF transmembrane conductance regulator (CFTR) into the respiratory epithelium of CF mice using amine carrying cationic lipids. This group followed up with a liposomal CF gene therapy trial which, although only partially successful, demonstrated the potential for this approach in humans (Caplen, NJ. *et al., Nature Medicine*, 1, 39-46, 1995). More recently other groups have investigated the potential of other cationic lipids for gene delivery, for example cholesterol derivatives (Oudrhiri,N *et al. Proc.Natl.Acad.Sci.* **94**, 1651-1656, 1997). This limited study demonstrated the ability of these cholesterol based compounds to facilitate the transfer of genes into epithelial cells both *in vitro* and *in vivo*, thereby lending support to the validity of this general approach.

These studies, and others, show that in this new field of research there is a continuing need to develop novel low-toxicity surfactant molecules to facilitate the effective transfer of polynucleotides into cells both *in vitro* for transfection in cell-based experimentation and *in vivo* for gene therapy and antisense treatments. The present invention seeks to overcome the difficulties exhibited by existing compounds.

The invention relates to the peptide-based gemini compounds comprising two linked chains:

$$\begin{array}{ccccccccc} Q^1 & - & P^1 & - & Y & - & P^2 & - & Q^2 \\ & & | & & & & | & & \\ & & R^1 & & & & R^2 & & \end{array}$$

each chain having:

(1) a positively charged hydrophilic head, $Q^1$ or $Q^2$, formed from one or more amino acids and/or amines;

(2) a central portion, $P^1$ or $P^2$, having a polypeptide backbone; and

(3) a hydrophobic tail, $R^1$ or $R^2$;

the central sections of each chain being linked together by bridge Y through residues in $P^1$ and $P^2$.

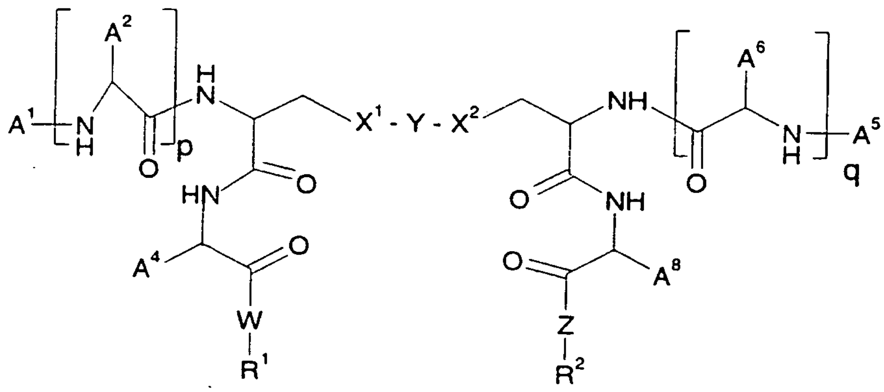
Preferably the central portion is made up of two or three amino acids, $P^a$ (optional), $P^b$ and $P^c$, in which:

$P^a$ is a D- or L- amino acid, preferably hydrophilic, such as threonine or serine,

$P^b$ is preferably D- or L- cysteine, serine or threonine, and

$P^c$ is preferably D- or L- serine or threonine and is linked to $R^1$ or $R^2$.

Preferred compounds of the present invention include compounds of the formula (I):



(I)

where:

$A^1$ and $A^5$ ,which may be the same or different, is a positively charged group formed from one or more amino acids or amines joined together in a linear or branched manner and preferably bonded by an amide (CONH) bond;

$A^2/A^6CH(NH)CO$, which may be the same or different, is derived from an amino acid, preferably serine;

p and q, which may be the same or different, is 0 or 1;

$X^1/X^2CH_2CH(NH)CO$, which may be the same or different, is derived from cysteine ($X^1/X^2 = S$), serine or threonine ($X^1/X^2 = O$);

$A^4/A^8CH(NH)CO$, which may be the same or different, is derived from serine or threonine;

Y is a linker group, preferably $(CH_2)_m$ where m is an integer from 1 to 6, most preferably 2, and may be a disulphide bond when $X^1$ and $X^2$ is each S;

$R^1$ and $R^2$ are $C_{(10-20)}$ saturated or unsaturated alkyl groups, and

W and Z are NH, O, $CH_2$ or S; or

a salt, preferably a pharmaceutically acceptable salt thereof.

Preferably, the compound is symmmetrical, that is $A^1$ and $A^5$ are the same, $A^2$ and $A^6$ are the same, $A^4$ and $A^8$ are the same, $R^1$ and $R^2$ are the same, and W and Z are the same.

Representative examples of $A^1/A^5$ include D- or L-amino acids selected from arginine, lysine, ornithine and histidine, preferably lysine, or amines such as spermine and spermidine. Up to seven amino acids and /or amines may be linked in a linear or branched chain. Prefered examples include groups having two or three lysines or ornithines or a combination of lysine, ornithine, arginine and histidine, for instance:

$$COCH(NHR)(CH_2)_4NHCO(NH_2)(CH_2)_4NH_2$$

or

$$COCH(NHR)(CH_2)_3NHCO(NH_2)(CH_2)_3NH_2$$

or

$$COCH(NHR)(CH_2)_4NHCO(NH_2)(CH_2)_3NH_2$$

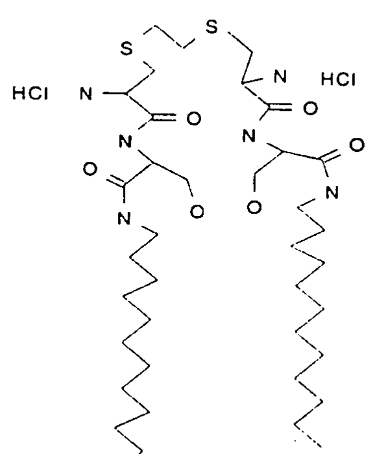
in which R is H or $NHCO(NH_2)(CH_2)_4NH_2$ or $NHCO(NH_2)(CH_2)_3NH_2$

Preferably, $-X^1-Y-X^2-$ is $-SCH_2CH_2S-$ or $-OCH_2CH_2O-$

Preferably, $R^1$ and $R^2$ is each a $C_{12}$-$C_{20}$ alkyl group, for instance $C_{12}$.

Preferably, W and Z is NH, thereby forming a further amide (CONH) bond.

Compounds of the present invention may be prepared from readily available starting materials using synthetic peptide chemistry well known to the skilled person. For prefered compounds of the present invention a useful intermediate is the compound:

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which is synthesised in a multi-stage process beginning, for instance, with the construction of the di-cysteine part and subsequently building up the hydrophilic head by attaching a serine moiety at the carboxyl group of each cysteine moiety, using standard peptide chemistry, and then attaching the hydrocarbon chains to the carboxyl group of the serine moiety using a standard amide forming reaction well known to those skilled in the art. This intermediate can then be taken through to compounds of formula (I) by further reaction at the nitrogens of the cysteine residues.

Another aspect of the invention relates to methods for using the peptide-based gemini compounds. Such uses include facilitating the transfer of oligonucleotides and polynucleotides into cells for antisense, gene therapy and genetic immunisation (for the generation of antibodies) in whole organisms. Other uses include employing the compounds of the invention to facilitate the transfection of polynucleotides into cells in culture when such transfer is required, in, for example, gene expression studies and antisense control experiments among others. The polynucleotides can be mixed with the compounds, added to the cells and incubated to allow polynucleotide uptake. After further incubation the cells can be assayed for the phenotypic trait afforded by the transfected DNA, or the levels of mRNA expressed from said DNA can be determined by Northern blotting or by using PCR-based quantitation methods for example the Taqman® method (Perkin Elmer, Connecticut, USA). Compounds of the invention offer a significant improvement, typically between 3 and 6 fold, in the efficiency of cellular uptake of DNA in cells in culture, compared with compounds in the previous art. In the transfection protocol, the gemini compound may be used in combination with one or more supplements to increase the efficiency of transfection. Such supplements may be selected from, for example:

(i) a neutral carrier, for example dioleyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289);

(ii) a complexing reagent, for example the commercially available PLUS reagent (Life Technologies Inc. Maryland, USA) or peptides, such as polylysine or polyornithine peptides or peptides comprising primarily, but not exclusively, basic amino acids such as lysine, ornithine and/or arginine. The list above is not intended to be exhaustive and other supplements that increase the efficiency of transfection are taken to fall within the scope of the invention.

In still another aspect, the invention relates to the transfer of genetic material in gene therapy using the compounds of the invention.

Yet another aspect of the invention relates to methods to effect the delivery of non-nucleotide based drug compounds into cells *in vitro* and *in vivo* using the compounds of the invention.

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"Amino acid" refers to dipolar ions (zwitterions) of the form $^{+}H_3NCH(R)CO_2^{-}$. They are differentiated by the nature of the group R, and when R is different from hydrogen can also be asymmetric, forming D and L families. There are 20 naturally occurring amino acids where the R group can be, for example, non-polar (e.g. alanine, leucine, phenylalanine) or polar (e.g. glutamic acid, histidine, arginine and lysine). In the case of un-natural amino acids R can be any other group which is not found in the amino acids found in nature.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Transfection" refers to the introduction of polynucleotides into cells in culture using methods involving the modification of the cell membrane either by chemical or

physical means. Such methods are described in, for example, Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). The polynucleotides may be linear or circular, single-stranded or double-stranded and may include elements controlling replication of the polynucleotide or expression of homologous or heterologous genes which may comprise part of the polynucleotide.

The invention will now be described by way of the following descriptions and examples.

## DESCRIPTIONS

**Description 1.** *Bisthioether* 3

A 1L 3-necked flask equipped with mechanical stirrer, reflux condenser and dropping funnel was flushed with $N_2$ directly into the flask through the condenser. A solution of 31.3g (0.20mole) L-cysteine.hydrochloride.x$H_2O$ (1) in 100ml (degassed ultrasonic for 10 minutes) was added to the flask. A degassed solution of 34g (0.40mole) $NaHCO_3$ in 300ml $H_2O$ was added, followed by the dropwise addition (30 minutes) of a degassed solution of 18.8g (8.6ml; 0.10mole) 1,2-dibromoethane (2) in 100ml EtOH. After another 30 minutes the mixture was heated to 65-70°C and stirred, still under $N_2$, for another 3 hours (within 1 minutes precipitation started). The mixture was cooled to 20°C, filtered, rinsed with 30ml $H_2O$ and with 100ml acetone (2x). After drying 19.4g white solid was obtained which still contained some free cysteine ($^1H$ NMR). The solid was suspended in 250ml 2.5%$NH_4OH$ and 25%$NH_4OH$ was added until a clear solution was obtained. To this solution 15mg KCN was added and the mixture was stirred for 30 minutes. The solution was acidified to pH6 using HOAc and stirred for 30 minutes whilst cooling to 5°C. The solid was collected, rinsed with $H_2O$ (100ml), acetone (2 x 100ml) and dried, yielding 18.1g (68%) 3 as a white solid.

**Description 2.** *Boc-L-Leucine* 5

19.7g (0.15mole) L-Leucine 4 was suspended in 200ml$H_2O$ and 6.75g (0.17mole) NaOH was added. The clear solution was cooled to <10°C and a solution of 36g (0.165mole) $(BOC)_2O$ in 100ml THF was added dropwise keeping T < 10°C (30 minutes). After stirring for 4 hours at room temperature the mixture was acidified to pH2 by adding 1N HCl. The mixture was extracted with EtOAc (250, 100 and 100ml), the combined organic layers were dried on $Na_2SO_4$ and evaporated, yielding 40g (>100%) 5 as a colorless oil which contained some THF but was used as such.

**Description 3.** *Boc-L-Leucine-OSuc* 7

40g crude 5 (max. 0.15mole) was dissolved in 400ml THF (distilled prior to use from LAH) under $N_2$. After addition of 17.3g (0.15 mole) N-hydroxysuccinimide (6) and 30.9g (0.15mole) DCC the mixture was stirred for 3.5 hours at room temperature. The mixture was filtered over a $P_2$ glassfilter, the filter was rinsed with 50ml THF and the filtrate was evaporated. The residue was dissolved in 400ml refluxing isopropylether, the solution was filtered hot and the filtrate was placed at 4°C for 20 hours. The solid was collected, rinsed with 50ml IPE and dried, yielding 32g (65%) 7 as a white solid.

**Description 4.** *Compound* 8

8.05g (30mmole) 3 was suspended in 200ml $H_2O$ and after addition of 8.3g (60mmole) $K_2CO_3$ the mixture was heated to get a clear solution. After cooling to room temperature a solution of 19.7g (60mmole) 7 in 200ml THF was added at once. The mixture was stirred at room temperature for 20 hours, followed by acidification to pH 6 (30% HCl). After filtration the filtrate was acidified to pH2-3 (30% HCl) and extracted with $CHCl_3$ (250, 100 and 100ml). The combined organic layers were once washed with brine (50ml), dried on $Na_2SO_4$ and evaporated, yielding 23g crude 8 as a white foam which was used as such.

**Description 5.** *Compound* 9

23g crude **8** was dissolved in 200ml EtOAc, cooled to 0°C and HC1-gas was bubbled through for 1 hour, followed by stirring at this temperature for another hour. The solid was collected, rinsed with ether and dried under vacuo over KOH. This yielded 15.4g (91%) **9** as a hygroscopic nearly white solid.

**Description 6.** *Compound* **10**

17.0g (30mmole) **9** dissolved in 250ml $H_2O$ and after cooling to < 10°C in an ice/waterbath 7.2g (180mmole) NaOH was added. After stirring for 10 minutes at room temperature a solution of 14.4g (66mmole) lauroylchloride in 50ml THF was added dropwise in 5 minutes. The mixture was stirred for 20 hours and extracted with hexame (2 x 150 ml). A 3-layer system formed and the lower 2 layers were acidified to pH 1-2 (1 N HC1) and extracted with ether (200, 100, 100 and 50ml). The combined ether layers were dried on MgSO4 and evaporated, yielding 24g (93%) **10** as an oil/foam. This material was used as such.

**Description 7.** *Compound* **11**

24g (max 27.5mmole) **10** dissolved in 400ml THF (distilled from LAH) under $N_2$. After addition of 6.33g (55mmole) N-hydroxysuccinimide (6) and 11.33g (55mmole) DCC the mixture was stirred at room temperature for 20 hours. The mixture was filtered over a large $P_2$ glassfilter, another 250ml THF was added to speed up filtration. The filter was rinsed with another 100ml THF. The filtrate was evaporated yielding 40g white solid and this crude material was recristallized from 400ml IPA. Stirred 1 hour 0°C and collected. After drying 22.3g (77%) **11** was obtained as white solid.

**Description 8.** *Compound* **13**

542mg (2.2mmole) H.Arg.$NH_2$.2HC1 (12) was dissolved in 15ml $H_2O$ and 304mg (2.2mmole) $K_2CO_3$ was added. A solution of 1.05g (1.0mmole) **11** in 15ml THF was added at once and the mixture was stirred at room temperature for 20 hours. Most of the

THF was evaporated and an oil formed in the waterlayer. This suspension was extracted with ether (6 x 50 ml), the combined ether layers were dried on $MgSO_4$ and evaporated, yielding 9500 mg (82%) **13 free amine** as a yellow solid. Treatment of this material with HCl-gas in $EtOAc/CH_2Cl_2$ gave **13** as a yellow solid.

**Description 9.** *Compound* **15**

5.25g (5.0mmole) **11** dissolved in 50ml THF (some heating was needed) and a solution of 1.31g (10.5mmole) Taurine (**14**) and 1.45 g (10.5mmole) $K_2CO_3$ in 50ml H2O was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and 300ml MeOH was added. Mixture placed at -20°C for 20 hours, solid collected, rinsed with MeOH and dried. This yielded 3.3g **15** contaminated with N-hydroxysuccinimide., which was combined with 700mg impure material of an other run. This 4.0g was recrystallized from 200ml MeOH + 50ml $H_2O$. Some solid was removed by filtration and the clear filtrate was placed at -20°C for 2 hours, the solid was colected, rinsed with MeOH and dried. This yielded 2.0g **15** as an off white solid. A 2$^{nd}$ crop of 800mg was obtained from the filtrate.

**Description 10.** *Compound* **16**

1.05g (1.0mmole) **11** was dissolved in 20ml THF and a solution of 275mg (2.2mmole) 2-aminoethylphosphonic acid and 300mg (2.2mmole) $K_2CO_3$ in 20ml $H_2O$ was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and the aqueous solution was freeze dried. This yielded a white solit which was recrystallized from 20ml MeOH and placed at -20°C for 20 hours. The solid was collected and dried yielding 150mg. $^1H$ NMR showed it to be N-hydroxysuccinimide. The filtrate was evaporated and the remaining yellow oil was dissolved in 10ml refluxing MeOH and after addition of 20ml IPA placed at -20°C for 4 hours. Solid was collected and dried, yielding 260mg.
The filtrate was evaporated, dissolved in 20ml EtOAc and placed at -20°C for 20 hours. The solid was collected.

**Description 11.** *Compound* 17

1.05g (1.0mmole) 11 was dissolved in 20ml THF and a solution of 310mg (2.2mmole) O-phosphocolamine and 300mg (2.2mmole) $K_2CO_2$ in 20ml $H_2O$ was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and the aqueous solution was freeze dried. The white solid was recrystallized from 20ml MeOH and placed at -20°C for 20 hours. The solid was collected, rinsed with MeOH and dried. This yielded 310mg white solid. No product. The filtrate was evaporated and the remaining yellow solid was dissolved in 10ml MeOH, 20ml IPA was added and the mixture was placed at -20°C for 20 hours. Solid collected, 20-30mg
Filtrate evaporated, residue dissolved in 25ml EtOAc, placed at -20°C and after 20 hours the solid was collected.

**Description 12.** *Boc-Glycine Boc-Glycine* 19

18.8g (0.25mole) Glycine 18 was suspended in 250ml $H_2O$ and 11g (0.275mole) NaOH was added. The clear solution was cooled to <10°C and a solution of 60g (0.165mole) $(BOC)_2O$ in 250ml THF was added dropwise keeping T < 10°C (20 minutes). After stirring for 20 hours at room temperature the mixture was acidified to pH1 by adding 1N HCl. The mixture was extracted with EtOAc (250, 100 and 100ml), the combined organic layers were dried on $MgSO_4$ and evaporated, yielding 47.5g (>100%) 5 as a colourless oil which contained some THF but was used as such.

**Description 13.** *Boc-Glycine-OSuc* 20

47.5g crude 19 (max. 0.25mole) was dissolved in 500ml THF (distilled prior to use from LAH) under $N_2$. After addition of 30g (0.26mole) N-hydroxysuccinimide (6) and 53.5g (0.26mole) DCC at < 10°C, the mixture was stirred for 20 hours at room temperature. Now the mixture was filtered over 1cm celite on a P2 glassfilter, the filter was rinsed with 200ml THF and the filtrate was evaporated. The crude material (56g) was recrystallized from refluxing isopropylether/THF (600ml 1:1), the solution was filtered

hot and the filtrate was stirred at 0°C for 3 hours. The solid was collected, rinsed with 50ml IPE and dried, yielding 16.4g (24%) **20** as a white solid. Filtrate evaporated and stored.

**Description 14.** *Compound* **21**

8.05g (30mmole) **3** was suspended in 200ml $H_2O$ and after addtion of 8.3g (60mmole) $K_2CO_3$ the mixture was heated to get a clear solution. After cooling to < 40°C a solution of 16.3g (60mmole) **20** in 200ml THF was added in 4 portions within 2 minutes. The mixture was stirred at room temperature for 72 hours, followed by acidification to pH 6 (30% HCl). After filtration the filtrate was acidified to pH 2-3 (30% HCl) and extracted with $CHCl_3$ (250, 100 and 100ml). The combined organic layers were once washed with brine (50ml), dried on $MgSO_4$ and evaporated, yielding 15.8g (90%) **21** as a white foam which was used as such.

**Description 15.** *Compound* **22**

15.8g (27mmole) crude **21** was dissolved in 300ml EtOAc and HCl-gas was bubbled through for 1 hour, followed by stirring at 0°C in an ice/waterbath and a solution of 12.2g (56mmole) lauroylchloride in 50ml THF was added at once. The mixture was stirred for 20 hours and extracted with hexane (2 x 100ml). The water layer was acidified to pH 1-2 (1 N HCl) and extracted with ether (3 x 150ml). A solid formed during extraction, which was collected and dried. This yielded 7.5g (39%) **23** white solid.

The filtrate was evaporated and the remaining slurry was stirred in 250ml $Et_2O$. An attempt to collect the solid failed and addition of 25ml MeOH gave a clear solution. This solution was placed at -20°C for 20 hours. The solid was collected, rinsed with ether and dried.

**Description 16.** *Compound* **24**

7.47g (10mmole) **23** dissolved in 200ml THF (distilled from LAH) under $N_2$. After addition of 2.53g (22mmole) N-hydroxysuccinimide (**6**) and 4.53g (22mmole) DCC the mixture was stirred at room temperature for 72 hours. The mixture was filtered over a 1cm layer celite on a large $P_2$ glassfilter (very slow). The filtrate was evaporated yielding 1.90g **24** as a foam.
The filer was rinsed with 300ml dioxane and the filtrate was evaporated yielding 6.9g **24** as a foam. Total yield 8.8g (94%).

**Description 17.** *Compound* **25**

542mg (2.2mmole) H.Arg.$NH_2$.2HCl (**12**) was dissolved in 15ml $H_2O$ and 750mg (5.4mmole) $K_2CO_3$ was added. A solution of 941mg (1.0mmole) **24** in 15ml THF was added at once and the mixtrue was stirred at room temperature for 20 hours. Most of the THF was evaporated and the waterlayer was extracted with EtOAc (2 x 50 ml), the combined EtOAc layers were dried on $MgSO_4$ and evaporated, yielding 150mg **25 free amine** as a foam. Both portions were combined and dissolved in $CH_2Cl_2$ and after the addition of 75ml EtOAc, HCl-gas was bubbled through for 1.5 hour. Now the mixture was cooled to 0°C and stirred for another 2 hours. Attempts to collect the solid failed, so 100ml ether was added and the mixture was stirred at room temperature for 20 hours. The solid was collected, rinsed with ehter and dried, yielding 520mg **25** as a slightly brown solid.

**Description 18.** *Compound* **26**

250mg (2.0mmole) taurine (**14**) was dissolved in 15ml $H_2O$ and 280mg (2.0mmole) $K_2CO_3$ was added. Now a solution of 941mg (1.0mmole) **24** in 15ml THF was added at once and the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated and the aqueous solution was freeze dried. The resulting white solid was recrystallized from 30ml MeOH, stirred 3 hours at 0°C and the solid was collected, rinsed with ether and dried.
This yielded 225mg **26** as a white solid.

The filtrate was partly evaporated and placed at -20°C for 20 hours. The solid was collected, rinsed with ether and dried, yieleing 210mg <u>26</u> as a white solid. Both portions were combined.

**Description 19.** *Compound* <u>**27**</u>

26.8g (0.1 mole) <u>3</u> was suspended in 300ml $H_2O$ and 9.6g (0.24mole) NaOH was added. A clear solution formed within 5 minutes, the mixture was cooled to < 10°C and a solution of 43.6g (0.2mole) $BOC_2O$ in 300ml THF was added dropwise in 30 minutes. The mixture was stirred at room temperature overnight. After addition of a solution of 2.5g (0.06mole) NaOH in 25ml $H_2O$ and 15g (0.07 mole) $BOC_2O$ in 75ml THF the mixture was stirred for another 18 hours.
The mixtue was acidified to pH 2 by adding 2N HC1 and after addition of 300ml brine, extracted with THF (3 x 400ml) and EtOAc (2 x 300ml) The combined organic layers were dried on $MgSO_4$ and evaporated, yielding 42g white solid. This solid was recrystallized from MEK/pentane, stirred at room temperature for 2 hours and placed at -20°C for 2 hours. The solid was collected and dried, yielding 37.8g (81%) <u>27</u> as a white solid.

**Description 20.** *Compound* <u>**28**</u>

8.9g (19mmole) <u>27</u> was dissolved in 300ml THF (from LAH) under $N_2$ and 4.37g (38mmole) <u>6</u> and 7.38g (38mmole) DCC were added. After stirring at room temperature for 18 hours the mixture was filtered over 1cm celite, the filter was rinsed with another 300ml THF and the filtrate was evaporated yielding 11.1g (88%) <u>28</u> as a white solid.

**Description 21.** *Compound* <u>**29**</u>

2.7g (20.6mmole) L-leusine (<u>4</u>) and 2.8g (20.3mmole) $K_2CO_2$ were dissolved in 100ml $H_2O$ and a suspension of 6.6g (10mmole) <u>28</u> in 50ml dioxane was added. The mixture was stirred at room temperature for 20 hours and most of the dioane was removed by

evaporation. The aqueous solution was extracted with 50ml ether and acidified to pH 1 by addition of 30% HC1. Now the mixture was extracted with $CHCl_3$ (150, 100 and 50ml), the combined organic layers were washed with brine (200ml), dried on MsSO4 and evaporated, yielding a white foam which was stripped with THF to get 7.5g (>100%) <u>29</u> as a solid white foam.

**Description 22.** *Compound* **<u>30</u>**

7.5g crude <u>29</u> (max. 10 mmole) was dissolved in 100 ml THF (from LAH) under $N_2$, 2.3g (20mmole) N-hydroxysuccinimide (<u>6</u>) and 4.12g (20mmole) DCC were added and the mixture was stirred at room temperature for 20 hours. The mixture was filtered over 1cm celite, the filter was rinsed with THF and the filtrate was evaporated, yielding 9.5g white foam which was recrystallized from 75ml IPA and placed at -20°C for 3 hours. The solid was collected but liquified immediately on the glass filter. The oily material was dissolved in 20ml THF and evaporated, yielding 6.5g (73%) <u>30</u> as a solid white foam.

**Description 23.** *Compound* **<u>31</u>**

6.5g (7.3mmole) <u>30</u> was dissolved in 100ml THF and after addition of 2.78g (15mmole) dodecylamine the mixture was stirred at room temperature for 18 hours. After evaporation a foam was obtained which was dissolved in 100ml $CHCl_3$. The solution was washed with $H_2O$ (2 x 75 ml), dried on MgSO4 and evaporated, yielding 7.5g (100%) <u>31</u> as a solid foam.

**Description 24.** *Compound* **<u>32</u>**

7.5g (7.3mmole) crude <u>31</u> was dissolved in 250ml EtOAc under heating and after cooling to room temperature HC1-gas was bubbled through for 2 hours. Stirring was continued at 0°C for 3 hours. The solid was collected, rinsed with ether and dried under vacuo over KOH. This yielded 4.0g (60%) <u>32</u> as a white solid.

**Description 25.** *Compound* 33

903mg (1.0mmole) 32 was dissolved in 10ml $H_2O$ under heating (gel formed), after cooling to room temperature, 80mg (2.0mmole) NaOH dissolved in 2ml $H_2O$ was added. A suspension was formed and THF was added until a clear solution was obtained. Now a solution of 572mg (2mmole) BOC-ß-alaOSuc (42) in 5ml THF was added and the mixture was stirred at room temperature for 5 hours. Most of the THF was removed by evaporation, another 30ml $H_2O$ was added and after stirring for another hour the solid was collected, rinsed with 10 ml $H_2O$ and dried. This yielded 1.0g (85%) 33 as an off white solid.

**Description 26.** *Compound* 34

1.0g (0.85mmole) 33 suspended in 25ml EtOAc and 25ml $CH_2Cl_2$ added to get a clear solution. HCl-gas bubbled through for 1.5 hour and stirred at 0°C for another 2 hours. No solid had formed so most of the $CH_2Cl_2$ was removed by evaporation and stirring at 0°C was continued for anothe 30 minutes. The solid was collected, partly by filtration (very slow), mainly be centrifugation. Total yield after drying 810mg (91%) 34 as a yellow solid.

**Description 27.** *Compound* 36

4.2g (40mmole) L-serine 35 and 5.53 (40mmole) $K_2CO_3$ were disslved n 300ml $H_2O$ and a suspension of 12.8g (max. 19mmole) 28 in 300ml THF was added. The mixture was stirred at room temperature for 72 hours and most of the THF was removed by evaporation. The aqueous solution was acidified to pH 1 by addition of 1N HCl. The mixture was extracted with $CH_2Cl_2$ + 15% MeOH (250, 100 and 100ml), the combined organic layers were dried on $MgSO_4$ and evaporated, yielding 8.5g (70%) 36 as a white solid foam which was used as such.

**Description 28.** *Compound* 37

8.5g (max. 13.2mmole) 36 was dissolved in 200ml THF (from LAH) under $N_2$ and after addition of 3.46g (30mmole) N-hydroxysuccinimide (6) and 6.2g (30mmole) DCC the mixture was stirred at room temperature for 24 hours. The mixture was filtered over 1cm celite, the filter was rinsed with 50ml THF and evaporated. This yielded 12.5g (>100%) 37 as a white foam which was used as such.

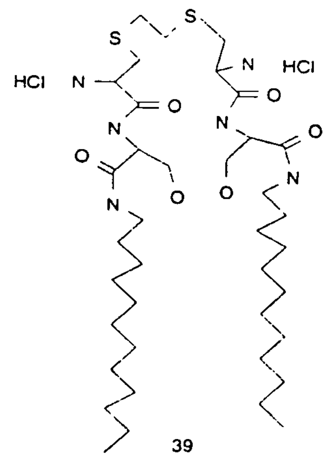
**Description 29.** *Compound* 38

12.5g crude (max 13.2mmole) 37 was dissolved in 200ml THF and stirred with 5.0g (27mmole) dodecylamine at room temperature for 48 hours. The THF was removed by evaporation and the residue was dissolved in 250ml $CHCl_3$ and extracted with brine (2 x 150ml). The combined brine layers were extracted with 50ml $CHCl_3$ and the combined $CHCl_3$ layers were dried on $MgSO_4$ and evaporated. This yielded 15.4g (>100%) 38 as a nearly white solid which was used as such.

## EXAMPLES

**Example 1.** *Compound* 39 2-amino-3-{2-[2-amino-2-(1-dodecylcarbamoyl-2-hydroxy-ethylcarbamoyl)-ethylsulphanyl]-ethylsulphonyl}-N-(1-dodecylcarbamoyl-2-hydroxy-ethyl-)-propionamide

15.4g (max. 13.2mmole) 38 dissolved in 400ml EtOAc and HCl-gas was bubbled through for 1.5 hour. The mixture was stirred at 0°C for 2 hours, the solid was collected, rinsed with ether and dried, yielding 9.9g (88%) 39 as a white solid.

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**Example 2.** *Compound* **40**

4.25g (5mmole) **39** was dissolved in 100ml $H_2O$ with heating and after cooling to <40°C a solution of 460mg (10mmole) NaOH in 10ml $H_2O$ was added. A suspension formed and THF was added until a clear solution was obtained (150ml). Next 2.86g (10mmole) BOC-ß-alaOSu (**42**) was added and the mixture was stirred at room temperature for 20 hours. Most of THF was removed by evaporation, another 100ml $H_2O$ was added and the mixture was stirred at 0°C for 3 hours. The solid was collected, rinsed with 20ml $H_2O$ and dried. This yielded 5.2g (93%) **40** as a nearly white solid.

**Example 3.** *Compound* **41**

5.2g (4.6mmole) **40** was dissolved in 100ml $CH_2Cl_2$ and 200ml EtOAc was added. HCl-gas was bubbled through for 1.5 hour and stirring was continued at 0°C for 2 hours. The solid was collected, rinsed with ether and dried, yielding 4.7g (100%) **41** as off white solid.

**Example 4.** *Compounds* **42** *and* **43**

After neutralization of compounds 34 and 41 using 2 eq. of NaOH in MeOH both compounds were treated with $(CH_2O)_n$ and $NaCNBH_3$ under $N_2$ for 18 hours. In both reactions complex mixtures were formed, probably due to alkylation on amide nitrogen as well.

**Example 5.** *Compound* 44

332mg (0.39mmole) 39 was dissolved in 15ml $H_2O$ under heating and after cooling to < 40°C a solution of 33mg (0.83mmole) NaOH in 1ml $H_2O$ was added. A white suspension formed and THF was added until a clear solution was obtained (25ml). To this solution 499mg (0.78mmole) BOC-Arg$(Z)_2$-OSu (47) was added and the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated and another 15ml $H_2O$ was added. After 2 hours stirring the solid was collected, rinsed with $H_2O$ and dried, yielding 700mg (98%) 44 as a white solid.

**Example 6.** *Compound* 45

100mg (0.05mmole) 44 was dissolved in 20ml HOAc and 500mg 10% Pd on Carbon (0.5mmole Pd) was added. The mixture was stirred under $H_2$ (5 bar) for 48 hours. The mixture was filtered over 1cm celite, the filter was rinsed with 10ml HOAc and the filtrate was evaporated. This yielded 100mg crude 45 as a green oil.

**Example 7.** *Compound* 46

100mg crude 45 (max 0.05mmole) was dissIved in 10ml $CH_2Cl_2$ and 10ml EtOAc was added. HCl-gas was bubbled through for 1 hour and the mixture was stirred 18 hours at room temperature. Most of the $CH_2Cl_2$ was removed by evaporation, 30ml ether was added and the mixture was stirred at 0°C for 1 hour. No crystalline material had formed so the mixture was evaporated, yielding 75mg crude 46 as a yellow oil.

**Example 8.** *Compound* 49

850mg (1.0mmole) **39** was dissolved in 20ml $H_2O$ and after 88mg (2.2mmole) NaOH was added a suspension formed. Now THF was added until a clear solution was obtained (30ml) and 974mg (2.2mmole) $BOC_2$LysOSuc (compound **48**) was added. After stirring at room temperature for 20 hours most of the THF was removed by evaporation, another 20ml $H_2O$ was added and the mixtrue was stirred for 2 hours. The solid was collected, rinsed with $H_2O$ and dried, yielding 1.35g (90%) **49** as a white solid.

**Example 9.** *Compound* **50**

500mg **49** was dissolved in 25ml $CH_2Cl_2$ and after addition of 25ml EtOAc HCl-gas was bubbled through for 1 hour, the mixture was stirred at 0°C for 1.5 hour. An attempt to collect the solid failed, 40ml ether was added and stirring was continued for 18 hours. The solid was collected, rinsed with ehter and dried, yielding 290mg **50** as a white solid.

**Example 10.** *Compound* **51**

425mg (0.43mmole) **41** was dissolved in 20ml $H_2O$ and 44mg (1.1mmole) NaOH was added. A suspension formed and THF was added until a clear solution was obtained (25ml). After addition of 487mg (1.1mmole) **48** the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated, another 20ml $H_2O$ was added and after stirring for 1.5 hour the solid was collected, rinsed with $H_2O$ and dried. This yielded 750mg **51** as a white solid.

**Example 11.** *Compound* **52**

250mg **51** was dissolved in 30ml $CH_2Cl_2$ and after addition of 30ml EtOAc, HCl-gas was bubbled through for 1 hour, the mixture was stirred at 0°C for 1.5 hour. The solid was collected, rinsed with ether and dried, yielding 120mg **52** as a white salt.

**Example 12.** *Compound* **57** (SucOSerLys$BOC_2$)

4.43g (10mmole) $BOC_2LysOSuc$ (**48**) was dissolved in 50ml THF and a solution of 1.16g (11mmole) L-serine and 1.52g (11mmole) $K_2CO_3$ in 50ml $H_2O$ was added immediately. The mixture was stirred at room temperature for 72 hours. Most of the THF was removed by evaporation and the remaining slurry was acidified to pH2 by the addition of 1M HCl and extracted with $CHCl_2$ (2 x 75ml). The combined organic layers were dried ($Na_2SO_4$) and evaporated, yielding **57** as a white solid foam which was used as such in example 43.

**Example 13.** *Compound* **54**

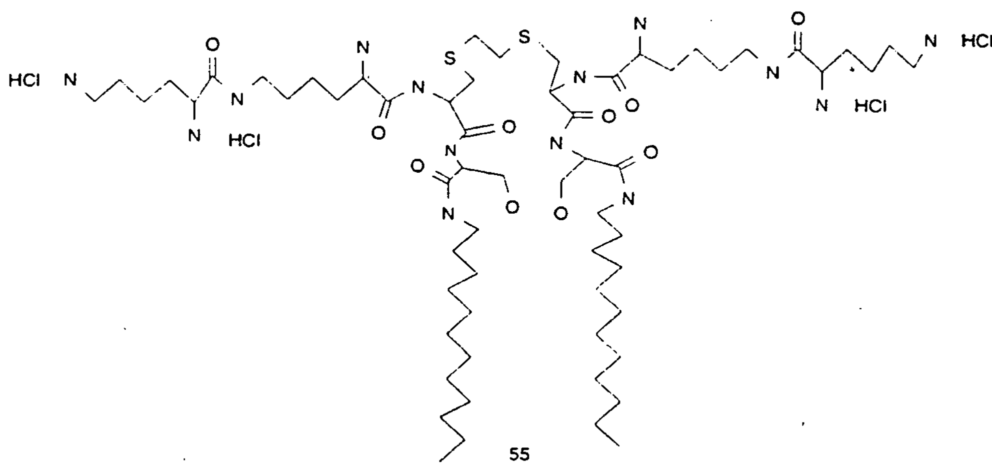
850mg (1.0mmole) **39** was dissolved in 30ml $H_2O$ and 88mg (2.2mmole) NaOH was added, followed by the addition of 30ml THF to get a clear solution. A solution of 1.5g (max. 2.3mmole) **57** in 30ml THF was added immediately and the solution was stirred at room temperature for 48 hours. Most of the THF was removed by evaporation, another 30ml $H_2O$ was added and stirring was continued for 1 hour. Because no solid had formed, the mixture was extracted twice with 75ml EtOAc/ether (2:1). The combined organic layers were dried ($Na_2SO_4$) and evaporated yielding the BOC-protected intermediate as a solid foam.

This foam was dissolved in 25ml $CH_2Cl_2$ and 50ml EtOAc was added. HCl gas was bubbled through the clear solution for 1 hour and stirring was continued at 0°C for another hour. The salt was collected, rinsed with ether and dried under vacuum, yielding 1.15g (85%) **54** as a slightly brown solid.

**Example 14.** *Compound* **55**

1.18g (1.0mmole) **50** was dissolved in 25ml $H_2O$ and 176mg (4.4mmole) NaOH was added, followed by the addition of 30ml THF to get a clear solution. 974mg (2.2mmole) **48** were added and the mixture stirred at room temperature for 48 hours. Most of the THF was removed by evaporation, another 50ml $H_2O$ was added and the mixture was stirred for 2 hours. Because no solid was formed the mixture was extracted with ether (2

x 100ml), the combined organic layers were dried ($Na_2SO_4$) and evaporated, yielding 2g of the BOC-protected intermediate as a solid foam. The foam was dissolved in 50ml EtOAc and HCl-gas was bubbled through the solution for 1 hour and stirring was continued at 0°C for another hour. The salt was collected, rinsed with ether and dried under vacuum, yielding 1.15g (76%) 55 as a nearly white solid.

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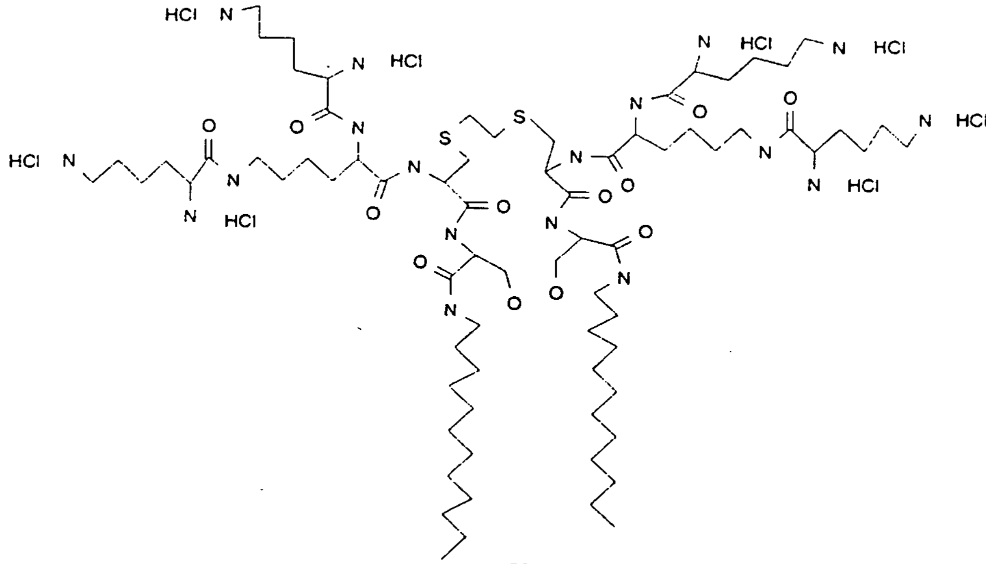
**Example 15.** *Compound 56*

Compound 56 was synthesised as for compound 55 except that 1.95g (4.4mmole) 48 was used. This yielded 1.1g (60%) 56 as an off white solid.



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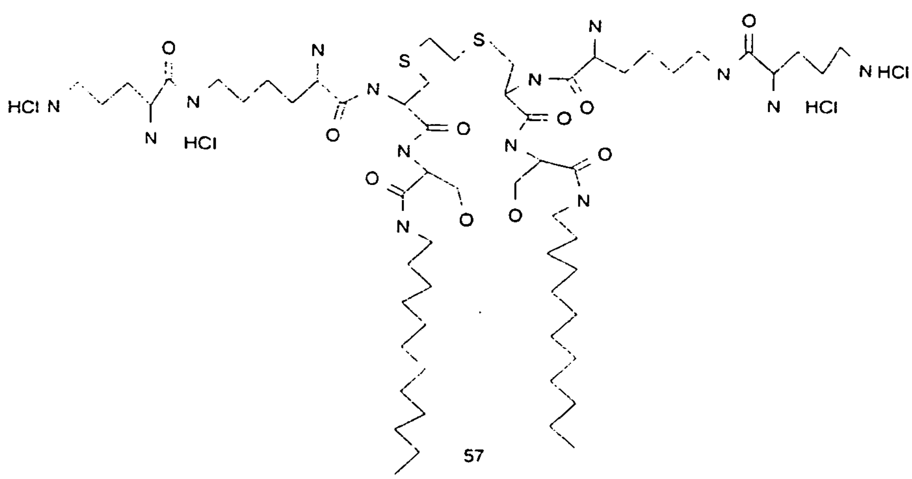
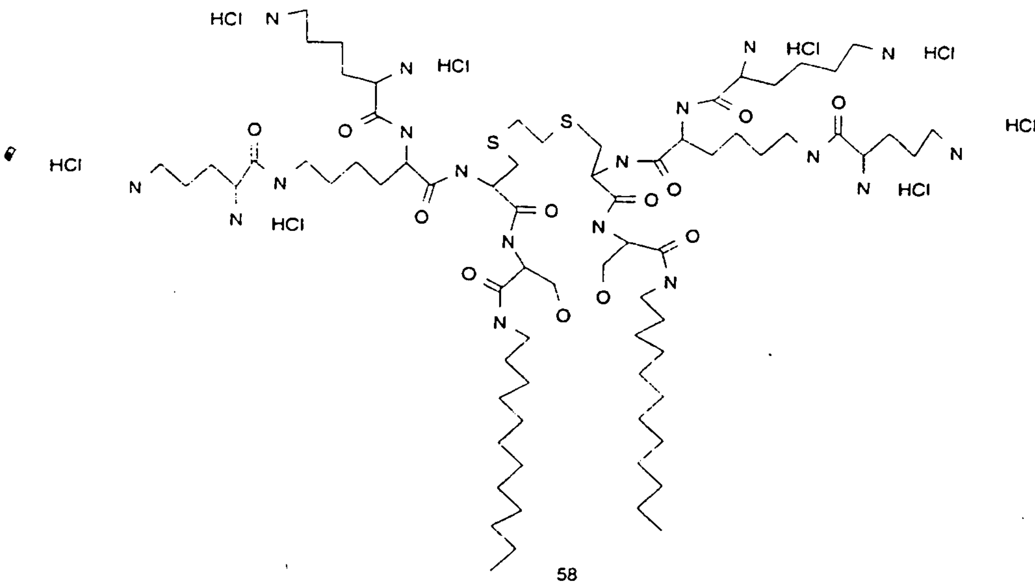
**Example 16.** *Compounds* __57__, __58__ *and* __59__

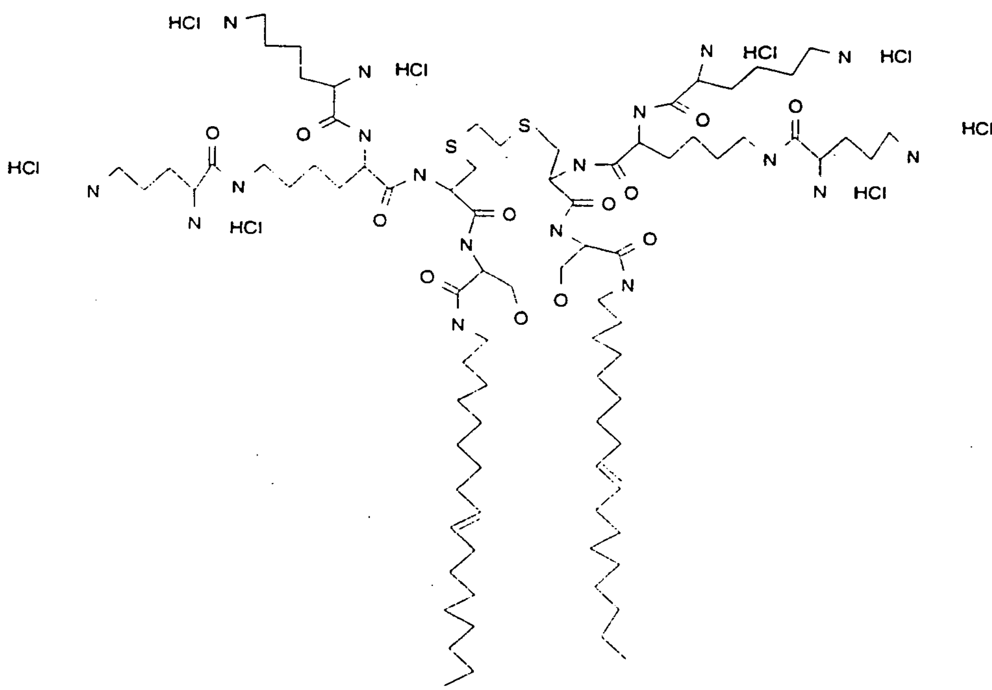
Compounds 57, 58 and 59 are synthesised in a similar manner to the compounds described above. Compound 39, or an intermediate equivalent to compound 39 but having different saturated or unsaturated hydrocarbon chains, is combined with an ornithine compound using synthetic peptide chemistry well known to the skilled person.



57



58



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It will be appreciated by a person skilled in the art that in the formulae shown in the examples above, the hydrogen atoms have been omitted from the N, C and O atoms, where appropriate, for clarity.

**Example 17. Transfection of recombinant plasmid expressing luciferase into HEK293 cells using peptide-based gemini compounds.**

All tissue culture reagents were obtained from Life Technologies Inc. HEK 293 cells were seeded at 2-3 x $10^5$ cells per well in Nunc six-well culture plates, 24 hours prior to transfection. The cells were seeded in 2mls Dulbeccos Modified Eagle medium containing Earles salts and supplemented with 10% v/v foetal bovine serum (= complete medium). The cells were grown at 37°C in 5% $CO_2$ in a humidified atmosphere. 6ug DNA ("luciferase control plasmid" from Promega Corp.) were dissolved in 100ul serum free medium (OPTI-MEM®). The peptide-based gemini compounds were made up at 1mg/ml in tissue culture grade water and then diluted in OPTI-MEM® to the appropriate concentration to a final volume of 100ul. The DNA and gemini solutions were mixed (to a total volume of 200ul; final concentrations of 5, 25, 50, 100, 150, 200, 250 and 300ug/ml) and left at room temperature for 15 minutes. The DNA/ gemini mix was placed onto the cells in each well and left in contact for 18-20 hours. The cells were then washed twice with phophate buffered saline prior to 1ml of fresh complete medium being added. Cells were incubated for a further 24 hours prior to lysis and luciferase activity assayed.

All luciferase activity assays were performed using the Canberra Packard (Berkshire, UK) Luclite kit according to the manufacturer's instructions with the exception that the cells in each well were resuspended in 1ml lysis buffer and 100ul aliquots mixed with 100ul of the luciferase substrate. The reaction mix was left for a 15 minutes adaptation period in the dark before counting for 5 minutes in a Top Count scintillation counter. Luciferase activity is measured as counts per second (CPS) from the scintillation counter. Four independent counts were taken per well.

Control transfections were set up with no DNA, $CaPO_4$, an anionic gemini compound (1) and the commercially available lipofection reagents LipofectAmine$^{TM}$ and Lipotaxi$^{TM}$ at the manufacturers recommended concentrations (10, 25, 50, 75, 125ug/ml and 175, 250, 325, 400 and 500ug/ml respectively).

The results (figure 1) clearly show that the cationic peptide-based gemini compounds (54), (55) and (56) are very efficient agents for facilitating the transfection of the luciferase plasmid into HEK293 cells at concentrations above 150ug/ml. In particular compound (54) peaks at 250ug/ml with a mean count (of 4 independent counts) of over 70,000cps. Compound (55) is most effective at 300ug/ml with an average count of about 45,000cps. Compound (56) is most effective at 200ug/ml with an average count of about 50,000cps. In contrast the 'no DNA' negative control gives a background count as do the anionic gemini (1) and the cationic geminis (50 and 52). The $CaPO_4$ transfection shows a very low count of about 2,000cps. In comparison figure 2 shows the results for the Lipofectamine transfections which at peak efficiency gave only 12,500cps (125ug/ml) and Lipotaxi 2,500cps (at 175ug/ml and 325ug/ml).

**Example 18. Transfection of recombinant plasmid expressing luciferase into CHO-K1 cells using peptide-based gemini compounds.**

CHO-K1 cells (ATCC: CRL-9618) were seeded into $T_{25}$-culture flasks (Corning-Costar Buckinghamshire, UK), at 7 x $10^5$ cells per flask, 24 hours prior to transfection. The CHO-K1 cells were seeded in 5 ml MEM alpha medium with ribonucleosides and deoxyribonucleosides and supplemented with 1x L-glutamine and 10 % v/v foetal bovine serum (complete medium). The cells were grown at 37°C in 5 % $CO_2$ in a humidified atmosphere.

For transfection, 5 ug DNA (luciferase control plasmid) was incubated with the gemini compounds in water (final volume 400 µl). The peptide-based gemini compounds were made up at 1 mg $ml^{-1}$ in tissue culture grade water and then diluted to the appropriate concentration to a total volume of 200 µl. Following a 30 minute room temperature incubation, 2.6 ml OPTI-MEM® medium was added and the solution placed on the cells. Following an overnight incubation at 37°C, the transfection solution was replaced with

complete medium and the cells incubated at 37°C. 24 hours post transfection the cells were detached from the flask and seeded into 96-well plates at a density of 0.5 x $10^5$ cells per well and incubated for a further 24 hours at 37°C. Luciferase reporter gene assays were performed according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany) approximately 48 hours post transfection. The plates were left for a 15 minutes adaption period in the dark before counting for 60 seconds in a TopCount NXT counter (Canberra Packard). An average of eight wells were counted per transfection.

Control transfections were set up with no DNA, an anionic gemini compound and the commercially available reagent LipofectAmine PLUS™.

The results, shown in figure 3, demonstrate that the cationic peptide-based compounds 54, 55, and 56 are very efficient agents for facilitating the transfection of the luciferase plasmid into CHO-K1 cells. Using the conditions described above, compound 54 peaks at 30 mM with a mean count in excess of 1.4 x $10^5$ counts per second (cps). Compound 55 is most effective at 30 mM with an average count of about 2.4 x $10^5$ cps. Compound 56 is most effective at 40 mM with an average count of about 1.9 x $10^5$ cps. In contrast negative controls gave a negligable count.

**Example 19. Transfection of recombinant plasmid expressing luciferase into CHO-K1 cells using peptide-based gemini compounds in combination with various supplements.**

The transfection ability of the gemini compounds could be further enhanced by the addition of a neutral carrier, for example, dioleyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289) or a complexing reagent, for example, PLUS compound (Life Technologies Inc.).

Figure 4 shows, for example, a 9-fold increase of luciferase activity at a 2:1 ratio of compound 55 and DOPE. Transfection mediated by compound 55 with DOPE in a 2:1 ratio and the addition of 11.6 ul of PLUS compound lead to a mean count of 6.5 x $10^5$ cps representing a 12-fold increase of luciferase activity in comparison to compound 55 alone. Incubation of the PLUS compound with the DNA and combination with compound 55 alone also lead to a 4-fold increase.

**Example 20. Use of peptide-based gemini compounds to facilitate adhesion of cells in culture to the culture flask.**

Using normal growth medium and culture conditions (RPMI plus 10% foetal bovine serum; 37°C, 5% $CO_2$ ) but with the addition of 50-60ug peptide-based gemini compound per well, it was observed that with the suspension cell line Jurkat, cells could attach to the bottom surface of the plastic culture vessel. In the absence of gemini compounds, the Jurkat cells grew in suspension.

**Brief description of the drawings**

Fig 1. Transfection of HEK 293 cells gemini compounds (1), (50), (52), (54), (55), (56). Bars represent the mean cps of four aliquots from duplicate wells.

Fig 2. Transfection of HEK 293 cells with Lipofectamine™ and Lipotaxi™ at manufacturers recommended concentrations. Bars represent the mean cps of four aliquots from duplicate wells.

Fig 3. Transfection of CHO-K1 cells with gemini compounds (54), (55), and (56). Bars represent the mean cps of 8 wells ± the standard error of the mean.

Fig 4. Transfection of CHO-K1 cells with gemini compound 55 with the addition of DOPE alone and/or PLUS. Bars represent the mean cps of 8 wells ± the standard error of the mean.

## CLAIMS

1. A peptide-based gemini compound comprising two linked chains:

$$\begin{array}{ccccccccc} Q^1 & - & P^1 & - & Y & - & P^2 & - & Q^2 \\ & & | & & & & | & & \\ & & R^1 & & & & R^2 & & \end{array}$$

each chain having:

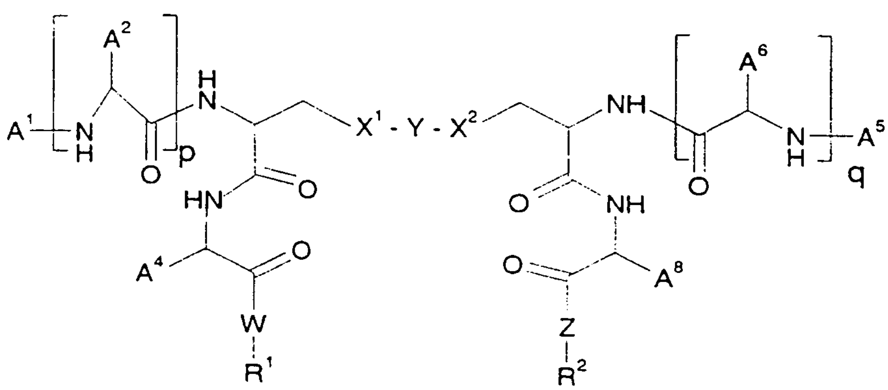
(1) a positively charged hydrophilic head, $Q^1$ or $Q^2$, formed from one or more amino acids and/or amines

(2) a central portion, $P^1$ or $P^2$, having a polypeptide backbone, and

(3) a hydrophobic tail, $R^1$ or $R^2$,

the central sections of each chain being linked together by bridge Y through residues in $P^1$ and $P^2$.

2. A peptide-based gemini compound according to claim 1 which has the formula (I):




(I)

where:

$A^1$ and $A^5$ ,which may be the same or different, is a positively charged group formed from one or more amino acids or amines joined together in a linear or branched manner;

$A^2/A^6CH(NH)CO$, which may be the same or different, is derived from an amino acid;

p and q, which may be the same or different, is 0 or 1;

$X^1/X^2CH_2CH(NH)CO$, which may be the same or different, is derived from cysteine ($X^1/X^2 = S$), serine or threonine ($X^1/X^2 = O$);

$A^4/A^8CH(NH)CO$, which may be the same or different, is derived from serine or threonine;

Y is a linker group or a disulphide bond when $X^1$ and $X^2$ is each S;

$R^1$ and $R^2$ are $C_{(10\text{-}20)}$ saturated or unsaturated alkyl groups, and

W and Z are NH, O, $CH_2$ or S; or

a salt thereof.

3. A peptide-based gemini compound according to claim 2 wherein the $A^1$ and $A^5$ groups are bonded by an amide (CONH) bond.

4. A compound according to claims 2 or 3 wherein $A^1/A^5$ are D- or L-amino acids selected from arginine, lysine, ornithine and histidine.

5. A compound according to claims 2 to 4 wherein $A^1/A^5$ have up to 7 amino acids linked in a linear or branched chain.

6. A compound according to claim 5 wherein $A^1/A^5$ have two or three lysines or ornithines or a combination of lysine, ornithine, arginine and histidine.

7. A compound according to any one of claims 2 to 6 wherein the amino acid from which the $A^2/A^6CH(NH)CO$ is derived is serine.

8. A compound according to any one of claims 2 to 7 wherein Y is $(CH_2)_m$, where m is an integer from 1 to 6.

9. A compound according to any one of claims 2 to 7 wherein Y is a disulphide bond when $X^1$ and $X^2$ is each S.

10. A compound according to claim 8 or 9 wherein m is 2.

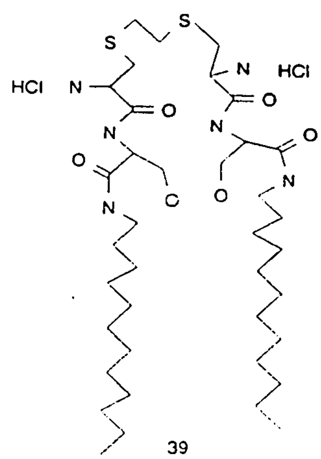
11. A compound according to any one of claims 2 to 10 wherein R is $C_{12}$ alkyl.

12. A compound according to any one of claims 2 to 11 wherein W and Z are NH.

13. A compound according to any one of claims 2 to 12 wherein the salt is a pharmaceutically acceptable salt.

14. A compound according to any one of claims 1 to 13 which is symmmetrical, that is $A^1$ and $A^5$ are the same, $A^2$ and $A^6$ are the same, $A^4$ and $A^8$ are the same, $R^1$ and $R^2$ are the same, and W and Z are the same.

15. Compound 39: 2-amino-3-{2-[2-amino-2-(1-dodecylcarbamoyl-2-hydroxy-ethylcarbamoyl)-ethylsulphanyl]-ethylsulphonyl}-N-(1-dodecylcarbamoyl-2-hydroxy-ethyl-)-propionamide, and derivatives thereof, compounds 40 to 58.

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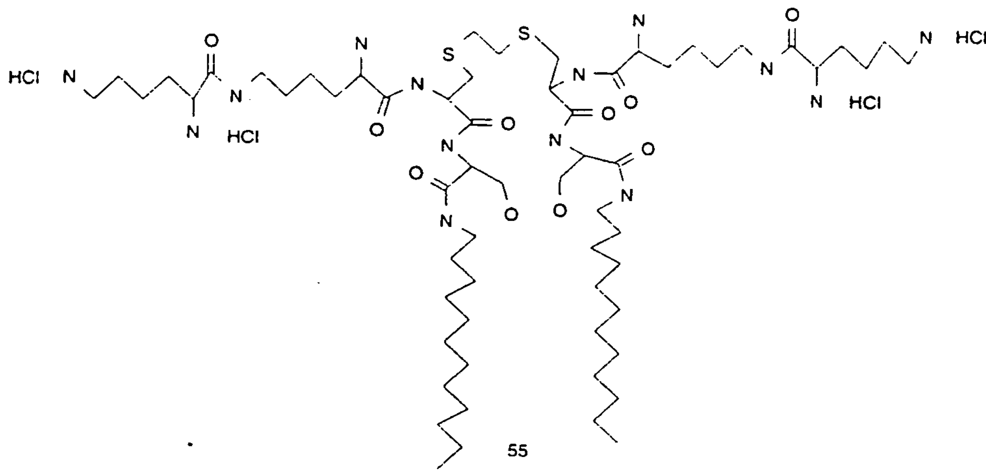
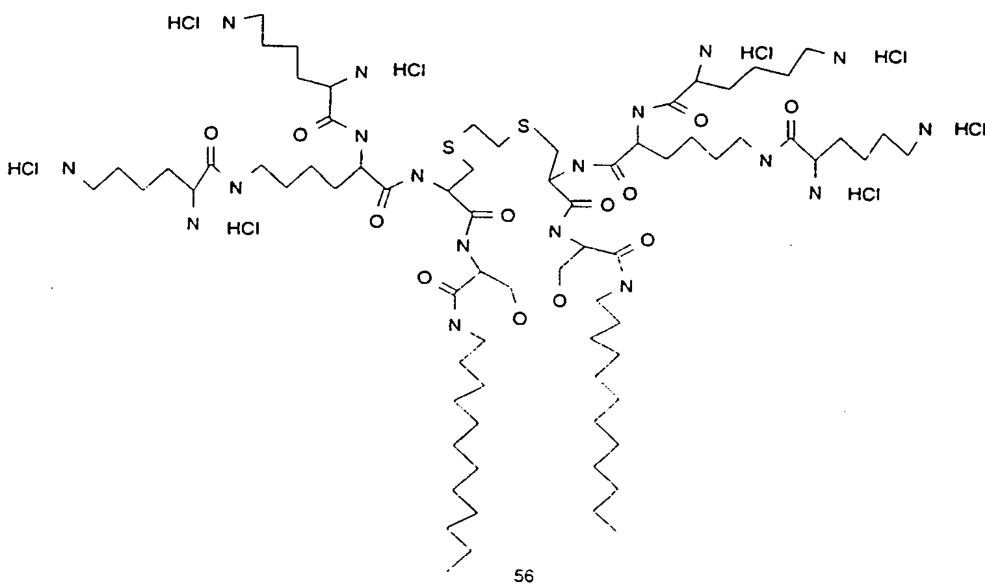
16. The compound:



55

17. The compound:



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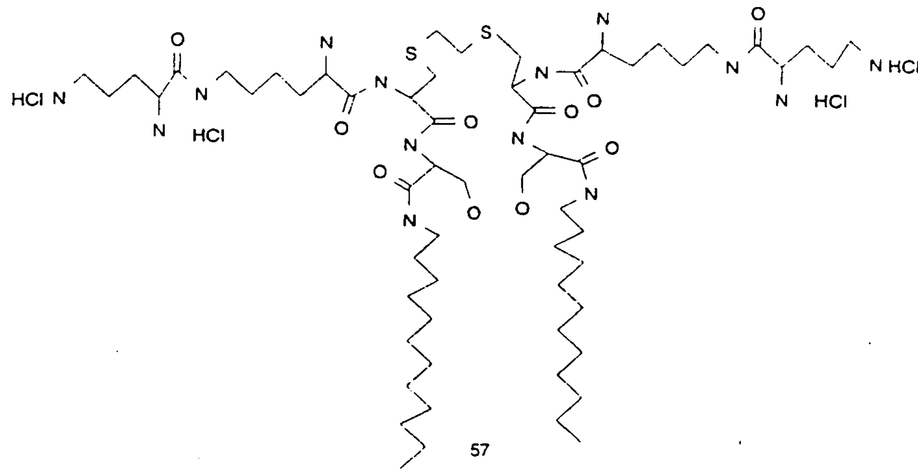
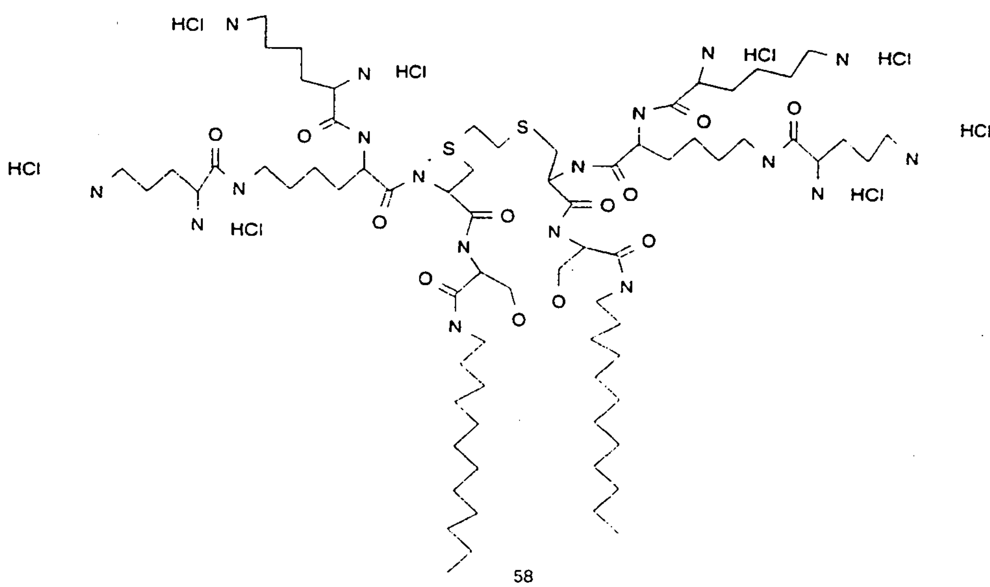
18. The compound:



57

19. The compound:



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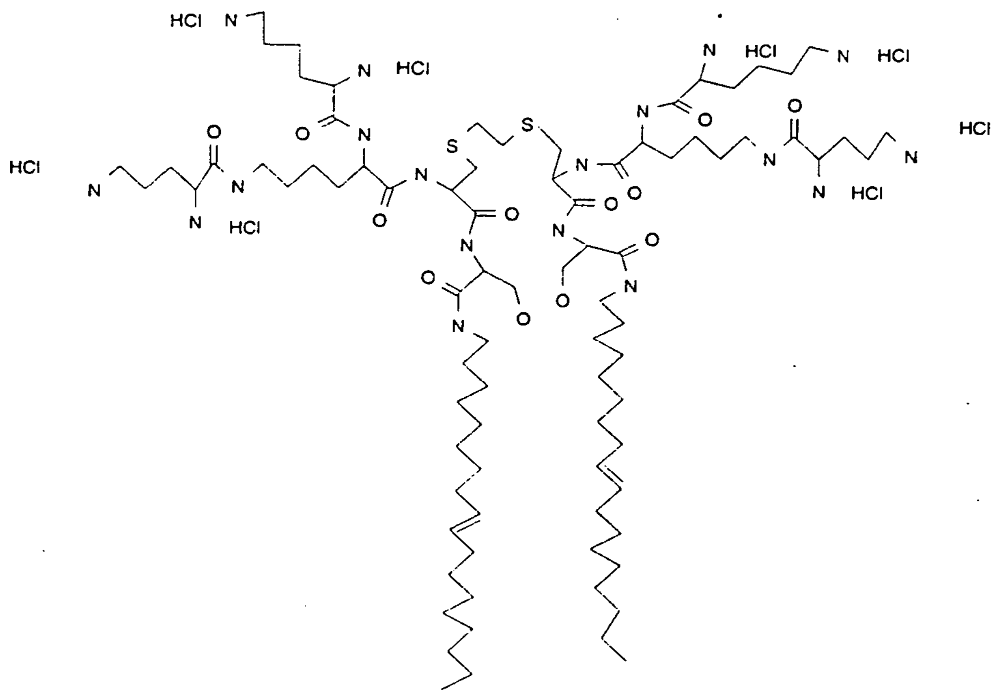
20. The compound:

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21. The use of a gemini-based peptide compound as defined in any one of claims 1 to 20 in enabling transfection of DNA or RNA or analogs thereof into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*.

22. The use of a peptide-based gemini compound according to claim 21 wherein the compound is used in combination with one or more supplements selected from the group consisting of:

(i) a neutral carrier; or

(ii) a complexing reagent.

23. The use according to claim 22 wherein the neutral carrier is dioleyl phosphatidylethanolamine (DOPE).

24. The use according to claim 22 wherein the complexing reagent is PLUS reagent.

25. The use according to claim 22 wherein the complexing reagent is a peptide comprising mainly basic amino acids.

26. The use according to claim 25 wherein the peptide consists of basic amino acids.

27. The use according to claim 25 or 26 wherein the basic amino acids are selected from lysine and arginine.

28. The use according to claim 26 wherein the peptide is polylysine or polyornithine.

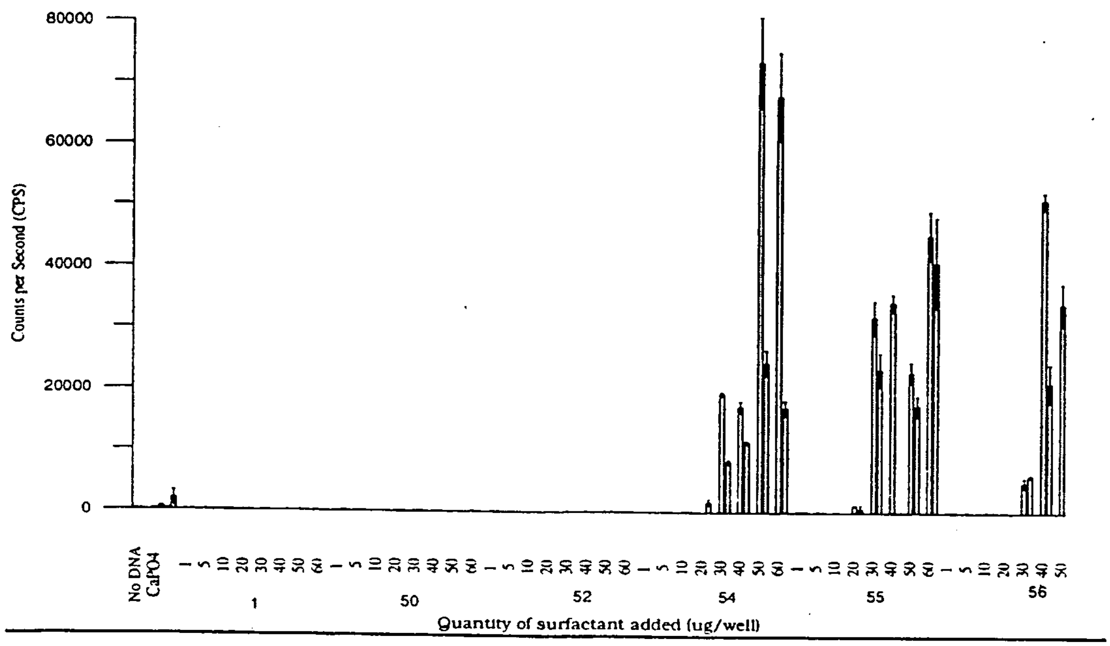
29. A method of transfecting polynucleotides into cells *in vivo* for gene therapy, which method comprises administering peptide-based gemini compounds of any one of claims 1 to 20 together with, or separately from, the gene therapy vector.

30. The use of a peptide-based gemini compound of any one of claims 1 to 20 to facilitate the transfer of a polynucleotide or an anti-infective compounds into prokaryotic or eukaryotic organism for use in anti-infective therapy.

31. The use of a peptide-based gemini compound of any one of claims 1 to 20 to facilitate the adhesion of cells in culture to each other or to a solid or semi-solid surface.

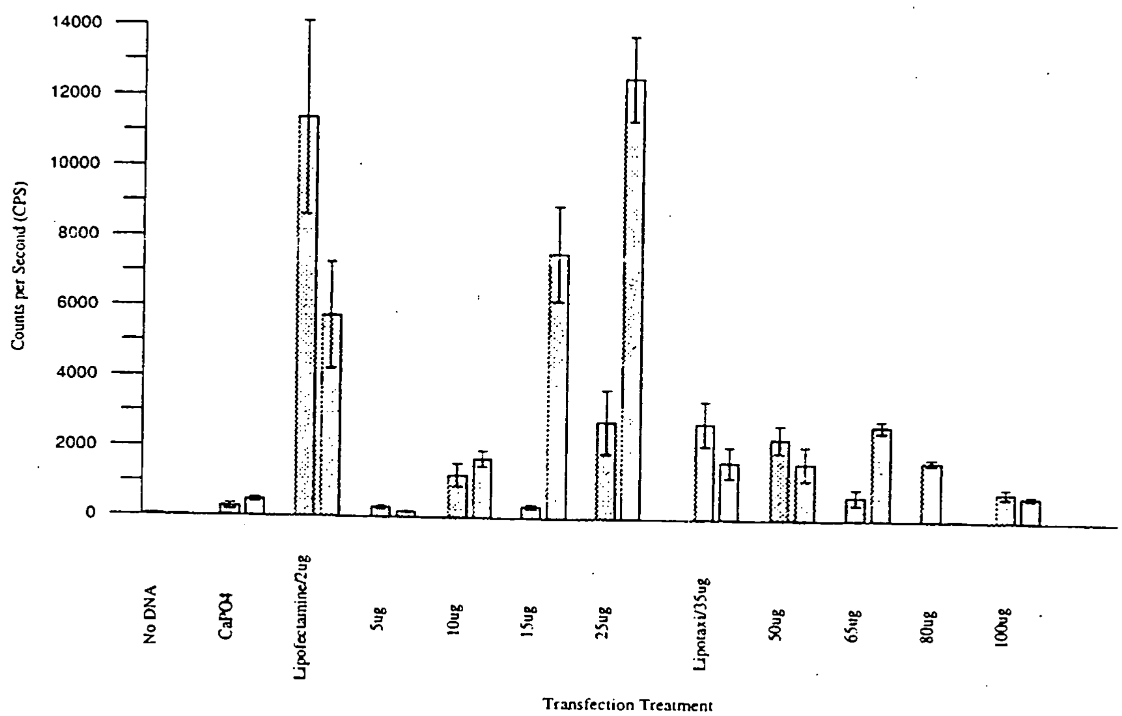
32. A process for preparing peptide-based gemini compounds of claim 1 or 2 which process comprises adding amino acids or peptides to 2-amino-3-{2-[2-amino-2-(1-dodecylcarbamoyl-2-hydroxy-ethylcarbamoyl)-ethylsulphanyl]-ethylsulphonyl}-N-(1-dodecylcarbamoyl-2-hydroxy-ethyl-)-propionamide.

# Fig. 1

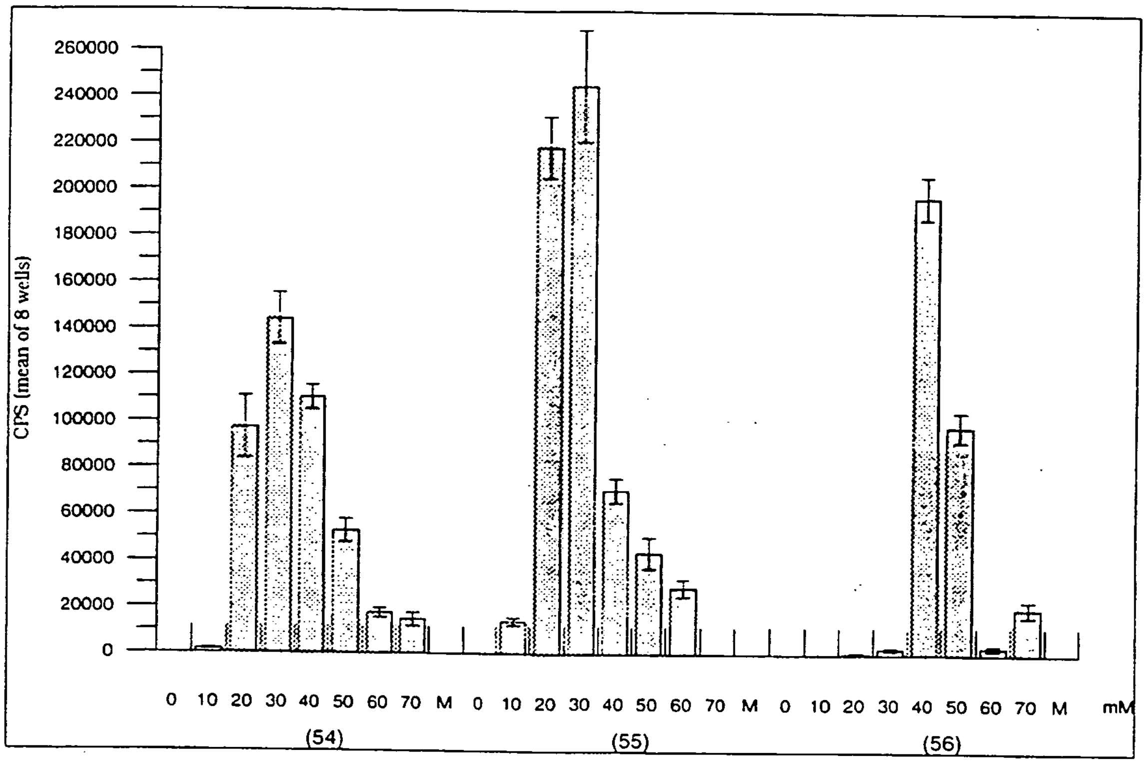


80000
60000
40000
20000
0
Counts per Second (CPS)
No DNA
CaPO4
1
50
52
54
55
56
Quantity of surfactant added (ug/well)

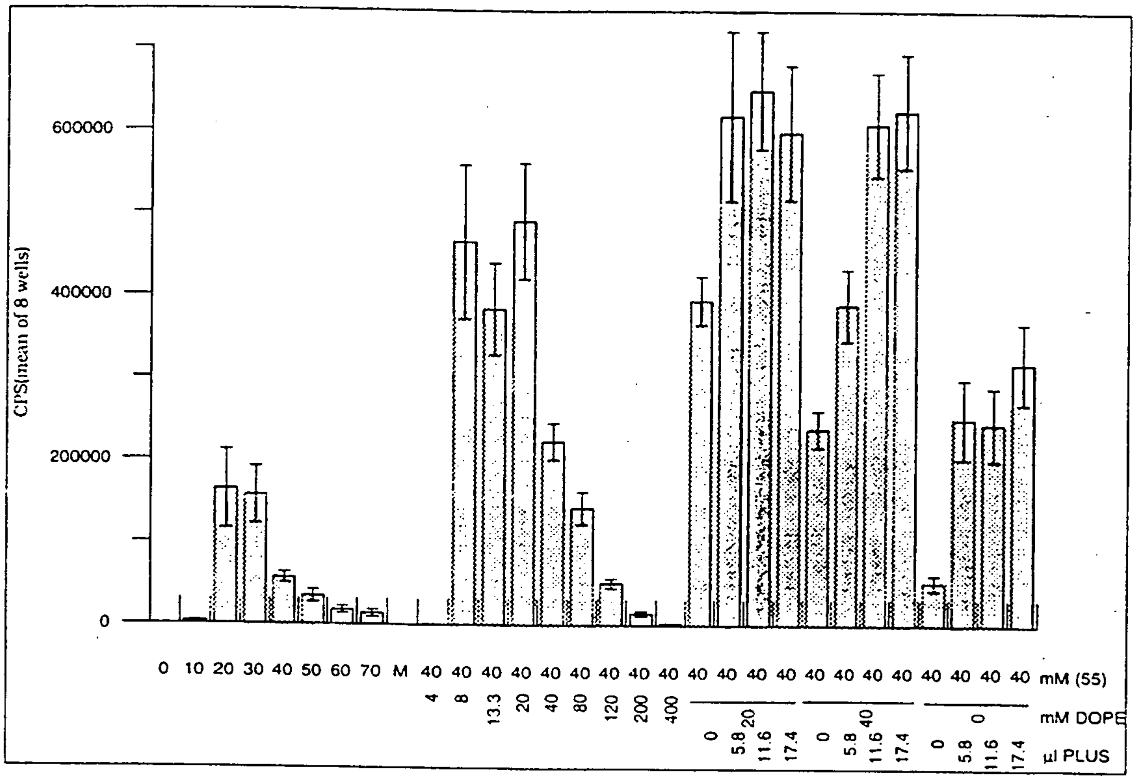
# Fig. 2


14000
12000
10000
8000
6000
4000
2000
0
Counts per Second (CPS)
No DNA
CaPO4
Lipofectamine/2ug
5ug
10ug
15ug
25ug
Lipotaxi/35ug
50ug
65ug
80ug
100ug
Transfection Treatment

# Fig. 3


CPS (mean of 8 wells)
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0 10 20 30 40 50 60 70 M 0 10 20 30 40 50 60 70 M 0 10 20 30 40 50 60 70 M mM
(54)
(55)
(56)

# Fig. 4


CPS(mean of 8 wells)
600000
400000
200000
0
0 10 20 30 40 50 60 70 M 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 40 mM (55)
4 8 13.3 20 40 80 120 200 400 20 40 0 mM DOPE
0 5.8 11.6 17.4 0 5.8 11.6 17.4 0 5.8 11.6 17.4 µl PLUS

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03652

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K5/02 C07K5/08 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y | see abstract | 21-30 |
| A | WO 96 25388 A (HUELS CHEMISCHE WERKE AG ;KWETKAT KLAUS (DE); KOCH HERBERT (DE); R) 22 August 1996 | |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 March 1999 | 29/03/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2, NL - 2280 HV Rijswijk, Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Cervigni, S |

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y | WADHWA M S ET AL: "PEPTIDE-MEDIATED GENE DELIVERY: INFLUENCE OF PEPTIDE STRUCTURE ON GENE EXPRESSION"<br>BIOCONJUGATE CHEMISTRY,<br>vol. 8, no. 1, January 1997, pages 81-88,<br>XP000642731<br>see abstract; table 1 | 21-30 |
| Y | EP 0 394 111 B (CENTRE NAT RECH SCIENT)<br>24 October 1990<br>see the whole document | 21-30 |
| A | WO 97 11682 A (UNIV PITTSBURGH)<br>3 April 1997 | |

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In onal Application No
PCT/GB 98/03652

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